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(54) Title: ANTIVIRAL OR ANTIFUNGAL COMPOSITION AND METHOD

(57) Abstract

Antiviral and antifungal compositions comprise a mixture of a ferrous salt and a plant extract of pomegranate rind, *Viburnum plicatum* leaves or flowers, tea leaves or maple leaves in aqueous solution.

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ANTIVIRAL OR ANTIFUNGAL COMPOSITION AND METHOD

5 Bacteriophage represent a diverse group of viruses that exert both positive and negative effects in microbiology. For example, in the dairy industry bacteriophages of the lactic acid bacteria represent a major source of starter culture failure with consequent poor-quality milk fermentations. A number of measures to prevent phage infection, including implementation of hygienic procedures, rotation of starter cultures, isolation of resistant mutants and formulation of media to suppress phage proliferation have been instituted with various degrees of success [1-4]. By contrast, coliphage have proven to be valuable monitors of faecal contamination in ground water and treated drinking water [5] and are effective viral models in studies of water quality and virucidal activity [6, 7]. Recent developments with genetically recombinant bacteriophage, containing either the bacterial luciferase lux genes or the ice nucleation gene ina, have established an additional value for phage in the rapid detection of bacteria in food and environmental samples [8-12]. In the above examples, the eventual destruction of bacteriophage is important either from the perspective of maintaining effective fermentation or from the viewpoint of good microbiological practice in the disposal of contaminated material. At present, the methods available for bacteriophage destruction such as heat and chemical disinfection [13, 14], have a significant impact on the viability and survival of associated bacteria. In the case of a sterilisation regime this is of little significance but the ability 10 30 35 to combat bacteriophage in industrial environments

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would benefit from an environmentally benign procedure that could differentially destroy the virus without damaging metabolically-active bacterial cells. One example is in the dairy industry as mentioned above.

5 Another concerns spraying plants which have symbiotic bacteria to control bacteriophage infection.

There are also many situations where it would be useful to be able to combat fungus without at the same time damaging metabolically-active cells. Plant extracts have been used against disease development in banana by fungi [15].

This invention provides an antiviral or antifungal composition comprising an effective concentration of a mixture of a ferrous salt and an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers, tea leaves and maple leaves.

An antiviral composition is one which combats, e.g. by preventing growth or preferably killing, virus such as bacteriophage. An antifungal composition is one which combats, e.g. by preventing growth and preferably by killing fungus and destroying its spores. Preferred compositions are antiviral or antifungal, but without at the same time substantially damaging metabolically active bacterial or other cells with which the virus or fungus is associated. Preferably the composition is an aqueous solution, i.e. one in which water is the sole or the main constituent solvent.

The composition comprises a ferrous salt, which may conveniently be ferrous sulphate. The nature of the anion is however not critical, provided that the salt is non-toxic to bacterial and other cells under the intended conditions of use and is water-soluble. A preferred ferrous salt concentration range is 0.1 mM to 0.1 M, particularly from 1 to 20 mM.

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The composition also contains an extract of a plant. These plant extracts may conveniently be prepared by boiling the comminuted plant part with water or other solvent. The resulting extract may be fractionated. It is probable that the extract contains one or more active components. Although such active components have remained refractive to purification, there is a uniform consistency in the extracted activity from different sources of the plant parts and from parts obtained at different times of the year.

10 The inventors have examined many different plants and have identified the following four as active:

15 - Pomegranate rind. Whole pomegranates can be comminuted and used, but the activity resides in the rind.

- Viburnum plicatum leaves or flowers.

- Tea leaves. These may be dried or green.

20 Other parts of the tea plant Camellia sinensis may be used.

25 - Maple leaves e.g. UK Acer pseudoplatanus or Canadian maple leaves, or more generally leaves or flowers of any part of the genus Acer.

The plant extract may be used as is, or diluted as appropriate, e.g. by a factor of up to 100. Effective compositions generally contain 10-90% by volume of the ferrous salt solution together with correspondingly 90-10% by volume of the concentrated or diluted plant extract. The composition should preferably be stored in the dark.

30 The invention also includes solid or liquid concentrates which on dilution with water gives compositions as described.

35 The invention also includes a method of controlling virus or fungus, which method comprises contacting the virus or fungus with an effective concentration of a ferrous salt and an effective

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concentration of an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers, tea leaves and maple leaves. These two components can be used in sequence in either order. Preferably, 5 however they are used mixed together as a composition as described above.

The virus or fungus to be controlled may be contacted with, e.g. immersed in, the composition, typically for a few seconds or minutes. Where the 10 virus or fungus is on a surface, the composition may be applied to the surface, e.g. by spraying or wiping.

The surface may be for example a work surface or a vessel or utensil used in a hospital or kitchen or an industrial environment, or an external surface of a 15 mammal e.g. a human or a plant. Or a solution containing virus or fungus may be mixed with a composition as defined.

As described in the examples below, ferrous sulphate in combination with selected plant extracts 20 effects complete destruction of a broad range of bacteriophage infecting diverse bacterial genera. In assays incorporating both bacteriophage and bacteria at 10^{12} and $10^9/\text{ml}$ respectively, the bacteriophage are entirely destroyed within two minutes without affecting 25 bacterial viability as measured by colony forming ability.

When used alone, ferrous sulphate has virucidal activity against phages, and also 30 bactericidal activity against some bacteria. This invention is based on the observation that ferrous sulphate, either alone or in combination with certain plant extracts, offers a potent broad spectrum virucidal activity. Since the activity of relatively 35 low levels of ferrous sulphate can be further potentiated by the addition of trace amounts of hydrogen peroxide (data not shown) it is likely that

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the mechanism of action involves, at least in part, a free radical system. A mechanism similar to that operating in the phagolysosome and defined by the Modified Haber-Weiss Reaction [17] is proposed.

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While resistance of bacterial cells may be effected through free radical scavenging and repair systems, it is clear that the plant extracts also have a role and that remains to be elucidated.

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Materials and Method

Bacteria and bacteriophage strains

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One Gram-positive Staphylococcus aureus NCIMB 8588 and two Gram-negative Salmonella typhimurium LT2 and Pseudomonas aeruginosa NCIMB 10548 bacteria were used. The bacteriophage with specificity for the above bacteria were NCIMB 9563 for Staph.aureus, Felix 01 for S.typhimurium [16] (obtained from Amersham International plc., Amersham, HP7 9NA, UK) and NCIMB 10116 and 10884 for Ps.aeruginosa. (All these bacteria and bacteriophage are available to the public.)

25

The bacterial cells were maintained on Tryptose Phosphate Broth (TPB; Oxoid) supplemented with 1% agar (TPA) and stored at 4°C with monthly subculture. When required, cells were resuscitated in 10 ml TPB (18h, 37°C) or an orbital shaker operating at 240 rpm. Appropriate bacterial dilutions were made in Lambda buffer (6 mM Tris, 10 mM MgSO₄.7H₂O, 50 µg/ml gelatin; pH 7.2). After treatment bacterial survival was determined by colony forming units (cfu) on TPA [18].

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Phage stocks were developed on their appropriate host strains by a plate lysis procedure essentially equivalent to growing bacteriophage Lambda-derived vectors [19]. Typical phage titres of 10¹²/ml

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were obtained. Phage stocks were maintained in Lambda buffer at 4°C and stocks retained a constant titre for several months.

5 Preparation of plant extracts

Pomegranate rind, Viburnum plicatum leaves or flowers, maple leaves and commercial tea leaves were blended in distilled water (25% w/v) and boiled for 10 min. After centrifugation (20,000 x g, 4°C, 10 30 min), supernatants were autoclaved (121°C, 15 min), cooled and stored at -20°C. A further purification of the pomegranate extract to a molecular weight cut-off of 10,000 Da was achieved by membrane ultra filtration and the filtrate stored as above.

15

Example 1

Preparation of Composition A

a. Preparation of 4.3 mM FeSO₄.7H₂O in Lambda-buffer.

First freshly prepare stock solution (0.53%) of FeSO₄.7H₂O (0.053 gm ferrous sulphate in 10 ml Lambda-buffer). After sterilisation by membrane filtration (0.45 µm, Whatman) prepare the final ferrous sulphate concentration of 4.3 mM by transferring 4.1 ml of the ferrous stock solution to a sterile test tube containing 14 ml of Lambda-buffer.

b. Preparation of 13% PRE (pomegranate rind extract): Mix 1.3 ml of stock solution of PRE (25% w/v) with 8.7 ml of Lambda-buffer.

Composition A was prepared 1-2 min prior to use by mixing 16.74 ml of 4.3 mM FeSO₄.7H₂O (a; yellow) with 8.265 ml of 13% PRE (b; yellow). After about 30 sec the colour of the mixture (a and b) changed greenish then to black. These mixtures of ferrous sulphate and PRE (a and b) should be protected

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from light.

Example 2

5 Virucidal Assay

Plant extracts were diluted 1:8 in Lambda buffer immediately prior to use and 300 µl of this diluted extract added to 700 µl of freshly prepared ferrous sulphate solution (4.3 mM FeSO₄.7H₂O; pH 6.5); 10 these mixtures should be protected from light. Bacteriophage (20 µl at 10¹² pfu/ml) or 20 µl of an appropriate dilution of bacteria (10⁹ cfu/ml) were placed in a sterile Eppendorf micro-centrifuge tube and 144 µl of the FeSO₄ solution or PRE solution or 15 composition A (Example 1) or of the above plant extract/FeSO₄ mixture added. After exposure of the bacteriophage or bacteria for 2 min at room temperature the activity of the mixture was neutralised by adding an equal volume of 2% (v/v) Tween 80 in Lambda buffer. 20 The number of bacteriophage or bacteria surviving the above protocol were measured by plaque forming units (pfu) or colony forming units respectively.

The results are set out in the following Tables 1 and 2. Table 1 indicates that pomegranate 25 rind extract alone has a slight virucidal activity against Pseudomonas phage but in combination with FeSO₄ there is a profound synergy. Eleven log reductions in plaque forming ability are obtained within 2 min. A similar synergy of virucidal action was achieved with 30 other plant extracts, leaves and flowers of Viburnum plicatum, maple leaves and tea leaves (Table 2).

Our particular interest in the above studies was to select agents with maximal virucidal activity whilst having little effect on bacteria. In this regard FeSO₄, either alone or in combination with 35 pomegranate rind extract, appears singularly

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successful. Table 1 shows that for each bacterium and phage combination tested there is a simple treatment that can eliminate bacteriophage activity without affecting bacterial visibility. The plant extracts 5 appear to serve a dual role in their interaction with FeSO₄. In the case of Pseudomonas phage they promote virucidal activity while for the Salmonella phage, which is completely inactivated by FeSO₄ alone, the pomegranate rind extract appears to provide a 10 significant protection to the bacterium.

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Table 1 The survival of different bacteria and bacteriophage species in 4.3 mM FeSO₄.7H₂O, 13% PRE (pomegranate rind extract; 20 min at 37°C) and Composition A (PRE + 4.3 mM FeSO₄.7H₂O; 2 min at room temperature) prepared in Lambda buffer.

	Microorganisms	Lambda Buffer	13% PRE	4.3 mM FeSO ₄ .7H ₂ O	Composition A*
Bacteriophage species (pfu/ml):					
	<u>Staphylococcus</u> NCIMB 9563	7×10^{11}	2×10^7	NIL	NIL
	<u>Salmonella</u> Felix O1	3×10^{12}	3×10^{12}	NIL	NIL
	<u>Pseudomonas</u> NCIMB 10884	5×10^{11}	5×10^9	5×10^{11}	NIL
	<u>Pseudomonas</u> NCIMB 10116	5×10^{11}	5×10^8	5×10^{11}	NIL
Bacteria species (cfu/ml):					
	<u>Staph.aureus</u> NCIMB 8588	3×10^9	2×10^6	3×10^9	5×10^6
	<u>S.typhimurium</u> LT2	2×10^9	2×10^9	NIL	2×10^9
	<u>Ps.aeruginosa</u> NCIMB 10548	4×10^9	4×10^9	1×10^7	4×10^9

* Escherichia coli phage M13mp18 was killed by Composition A, whilst E.coli JM101 was unaffected.

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Table 2. Effect of 25% solutions of different plant extracts in the presence of 4.3 mMol ferrous sulphate on the inactivation within 3 minutes of Ps.aeruginosa bacteriophage NCIMB 10116.

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	25% Plant extract solution (%v/v)	PFU/ml
10	Lambda Buffer (control)	1.2×10^{10}
	Commercial tea leaves	Nil
15	<u>Viburnum plicatum</u> leaves or flowers	Nil
	Maple leaves	Nil

20

An antiserum was raised in rabbits against the Pseudomonas phage. A classic precipitin band was obtained with a double-immunodiffusion assay of phage antigen against this antiserum. No such precipitin band was obtained, however, when the phage had been treated with the virucidal agent, nor if the antiserum had been raised against treated phage.

Treated phage were further examined by electron microscopy. No recognisable phage morphological structures remained in any of the twenty fields examined. The result of the further tests are consistent, therefore, with a complete destruction of the bacteriophage structure by the virucidal composition, to an extent that antigenic determinants are lost.

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Example 3

Antifungal Test

Apple leaves infected with powdery mildew
5 were immersed in Composition A (Example 1) for about 60 seconds or until the leaves were completely wetted. The leaves were then observed daily. The powdery mildew was completely inhibited within 24 hours and no mildew lesions were present on the treated apple leaves
10 as they hardened off.

A further microscopic test of treated powdery mildew spores and mycellium with Composition A showed that both spores and mycellium were destroyed after 1 hour at room temperature. Composition A neutralised by
15 2%. TW80 was not effective however.

Example 4

Composition A was tested under the conditions described in Example 2 for activity against poliovirus, herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus type 1. 20 µl of an appropriate dilution of virus was mixed with 144 µl of composition A. After exposure, the virucide was neutralised by addition of a 2% v/v solution of Tween 80 in lambda buffer. In cell culture assays using HT-29 cells (for poliovirus), MRC-5 cells for HSV-1 and IIUT-78 cells for HIV-1 there was complete reduction of infectivity of initial inocula of $1-4 \times 10^6$ virus. There was no apparent cytopathic effect on MRC-5 cells or HT-29 cells.
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CLAIMS

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1. An antiviral or antifungal composition comprising an effective concentration of a mixture of a ferrous salt and an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers and maple leaves.
- 10 2. A composition as claimed in claim 1, which is an aqueous solution.
3. A composition as claimed in claim 2, wherein the ferrous salt concentration is 0.1 mM to 0.1 M.
- 15 4. A composition as claimed in claim 2 or claim 3, wherein the plant extract is used at a dilution of 1 to 100 times.
- 20 5. A composition as claimed in any one of claims 2 to 4, wherein the iron salt is ferrous sulphate used at a concentration of 1 - 20 mM and the plant extract is pomegranate rind extract.
- 25 6. A solid or liquid concentrate which on dilution with water gives a composition according to any one of claims 2 to 6.
7. A method of controlling virus or fungus, which method comprises contacting the virus or fungus with an effective concentration of a ferrous salt and an effective concentration of an extract of a plant selected from pomegranate rind, Viburnum plicatum leaves or flowers, tea leaves and maple leaves.
- 30 8. A method as claimed in claim 7, wherein the ferrous salt is used as a 0.1 mM to 0.1 M aqueous solution.

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9. A method as claimed in claim 7 or claim 8,
wherein the virus or fungus is on a surface and the
ferrous salt and the plant extract are applied to the
surface.

5 10. A method as claimed in any one of claims 7 to
9, wherein the virus or fungus is contacted with an
aqueous composition comprising a mixture of the ferrous
salt and the plant extract.

10 11. For use as an antiviral or antifungal agent,
a composition containing a mixture of an effective
concentration of a ferrous salt and an effective
concentration of an extract of a plant selected from
pomegranate rind, Viburnum plicatum leaves or flowers,
tea leaves and maple leaves.

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INTERNATIONAL SEARCH REPORT

Internat'l Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbol)
IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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